# The Role of β2 Spectrin in the canonical TGF-β/SMAD pathway in the adult heart

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in the undergraduate colleges of The Ohio State University.

By

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Acknowledgements:

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This work is highly collaborative, and I am extremely grateful for everyone that had a hand in its production. Due to the pandemic, there were certain experiments that were limited in scope due to scarce resources and limited production. Additionally, undergraduate students were limited in the laboratory for eight months that caused a further setback to research. I am thankful for Dr. Smith, Dr. Mohler, and Dr. Hund and the rest of the leadership at the Davis Heart and Lung Research Institute for prioritizing the health and safety of students and faculty in this challenging time.

Abstract:

Heart failure affects over 6 million Americans with an increasing need to prevent the progression of this disease. The TGF-β pathway is a signal transduction pathway involved in cell proliferation, apoptosis, fibrosis, and more, both in homeostasis and in disease states. β2 spectrin is a cytoskeletal protein involved in maintaining cellular structural integrity and cardiac excitability as well as functions as a key adaptor protein for SMAD3 and SMAD4. While there are data describing complete knockdown of β2 spectrin and its effects in the TGF-β/SMAD pathway from an embryological/developmental perspective, there is a *knowledge gap* regarding the function of β2 spectrin in the adult heart. Using a novel transgenic model, we sought to define the importance of β2 spectrin in the TGF-β/SMAD pathway in the adult heart. I ***hypothesized*** that β2 spectrin is a *key adaptor protein* in the TGF-β/SMAD pathway required for downstream transcription of fibrotic genes, and *chronic* TGF-β stimulation leads to increased intracellular calcium and thus calpain activity which cleaves β2 spectrin leading to HF. Our preliminary data using immunoblotting analysis demonstrated that there were significantly decreased expression of Collagen 1, the most significant fibrotic protein, as well as TGF-β1, and TGF-β2, suggesting that the canonical TGF-β is greatly affected. Inversely, it was seen that there are no significant changes in SMAD2, SMAD3, SMAD4 expression, but increased expression of SMAD7. Together, these data indicate that β2 spectrin does play a role in the TGF-β/SMAD pathway, but there is more work that must be done in order to elucidate the complete mechanism. Further *in vitro* studies in HL-1 cells and cardiac fibroblasts as well as the use of a HF transverse aortic constriction (TAC) mouse model would be ideal future studies to

mechanistically determine if calpain inhibition could be used to minimize and reduce fibrosis which would lead to improved outcomes in heart failure.

Introduction:

Heart failure (HF) is characterized by the heart lacking the ability to consistently meet the body's blood and oxygen demands. Over 6 million Americans suffer from HF, and with that number expected to grow, it is imperative that underlying mechanisms are elucidated and new clinical targets identified.[1] During the initial stages of heart failure, the heart undergoes both electrophysiological and fibrotic ventricular remodeling.[2] While initially beneficial, this compensatory remodeling creates a feedforward cascade of worsening HF.[3] Hence, there is significant research into limiting the rate of remodeling as well as the potential for reversing remodeling in medical care.

The TGF-β pathway is one of the primary fibrotic pathways and is often implicated in disease states. This signal transduction pathway has many potential targets for intervention due to its expansive nature. The fibrotic pathway follows the canonical SMAD signal transduction and β2 spectrin is an adaptor protein for SMAD3/4. β2 spectrin is the most prevalent non-erythrocytic spectrin isoform and is found in all nucleated cells. Besides serving as a primary cytoskeletal scaffolding protein, β2 spectrin is also vital for post-translational localization and signal transduction. In addition to the interconnectedness in this pathology, the relationship has been demonstrated in several tissues and disorders. [Reviewed in 4,5]

The TGF-β superfamily consists of TGF-β, bone morphogenetic proteins (BMPs), growth and differentiation factors, as well as the activin and inhibin subfamilies. The TGF-β pathway is

involved in cell proliferation, differentiation, and apoptosis, accomplished primarily through the intracellular signal transduction of the SMAD proteins. [6,7] At the tissue level, this superfamily concerns itself with regulation of tissue homeostasis and immune system function. [8,9] There are further intracellular signaling pathways that TGF-β regulates, coined non-canonical TGF-β signaling or the SMAD independent signaling, such as PI3K-AKT pathway, Rho pathway, JNK pathway, p38 MAPK pathway, and Ras-ERK-MAPK pathway. [10,11] The TGF-β cytokines function through binding to transmembrane Ser/Thr kinase receptors (type I and II). The SMAD proteins, the known proteins acting in concert with β2 spectrin, can be classified into receptor-activated SMADs (R-SMADs) that are SMADs 1-3,5,8 and inhibitory SMADs that are SMAD6 and SMAD7.[7] In the transcriptional TGF-β pathway, β2 spectrin is phosphorylated, associating β2 spectrin with SMAD3/4 (and specifically the N terminus of SMAD3); a heterocomplex is formed of SMAD2/3/4 and it is trafficked into the nucleus for transcription with a variety of transcription factors. [12] These transcription factors are necessary for the highly specific DNA transcription as SMADs maintain a low affinity for DNA, $K_d = 1 \times 10^{-7}$ M. [13]

β2 spectrin is paramount for maintaining regular heart physiology. β2 spectrin is the primary spectrin maintaining structural integrity in the heart. β2 spectrin helps preserve regular membrane excitability and plays a key role in maintaining normal cardiac development during embryogenesis. Additionally, β2 spectrin forms complexes with numerous ankyrin and actin proteins. [Reviewed in 14] The β2 spectrin/ankyrin-B complex is crucial for both $Na^+/K^+$ ATPase and $Na^+/Ca^{2+}$ exchanger localization and abnormalities with this complex are linked to conditions such as atrial fibrillation. In addition to the electrophysiological dysfunction, lack of β2 spectrin in the heart leads to an accelerated HF phenotype.[15,16]

Calpains are proteolytic enzymes that are ubiquitously expressed, calcium-dependent cysteine proteases. In cardiac pathology, it has been demonstrated that calpains are activated following myocardial infarction and are responsible for the mechanically induced reperfusion injury. [Reviewed in 17] Furthermore, there has been compelling evidence in rodent models that calpain inhibition could be a therapeutic target for HF and myocardial dysfunction.[16] For these reasons, calpain is critical in cardiovascular disease. Mechanistically and of our interest, Calpain-2 is responsible for calcium-dependent cleavage of β2 Spectrin.[14] It has been shown that TGF-β stimulation increases basal intracellular calcium, an indirect mechanism by which calpain could be activated.[19] With this information in mind, I hypothesized that *chronic* TGF-β stimulation will result in increased calpain activation that will cleave β2 Spectrin away from stabilizing the cytoskeleton to a subsequent role as an adaptor protein in the TGF-β/SMAD pathway, leading to increased intranuclear β2 Spectrin, cardiac fibrosis, and HF progression.

While there have been foundational studies in hepatocytes and renal fibrosis elucidating the function of β2 Spectrin in the TGF-β/SMAD pathway, there is no literature encompassing the nature of this relationship in the adult heart. Further, there are little data understanding if calpain is activated by TGF-β in cardiomyocytes and how the potential activation results in proteolytic cytoskeletal cleavage.

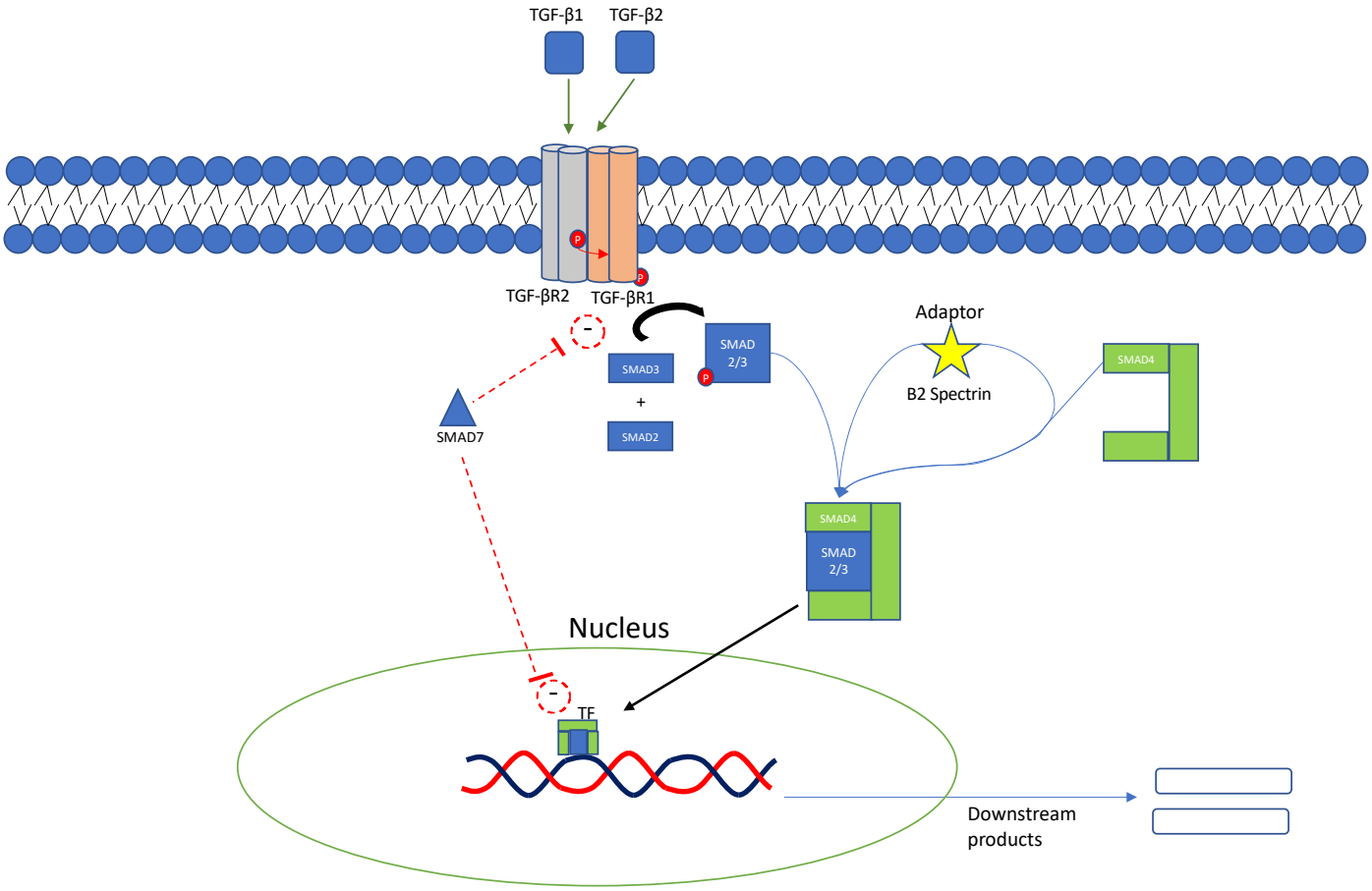



Figure 1. β2 Spectrin functions as an adaptor protein for SMAD3 and SMAD4. This allows for the SMAD2/3/4 complex to form and translocate into the nucleus where it functions as a transcription factor.

Methods:

*Animal Studies:*

Procedures followed were approved and in accordance with institutional guidelines at Ohio State University. The β2 Spectrin cKO mouse model was generated through the introduction of 2 LoxP sites flanking exon 3 of the gene then crossing the floxed mice with mice expressing Cre under the cardiac promoter a-myosin heavy chain (Figure 2). This produced a cardiac-specific loss of β2 spectrin in the mice that was validated through PCR and gel electrophoresis as well as western blot analysis and confocal microscopy (not shown).

*Whole Heart Tissue extraction and Lysate Preparation:*

Male and female mice that were eight-to twelve-weeks old received an intraperitoneal dose of anticoagulant heparin (100cc/kg, Thermo Fisher) 20 minutes prior to heart extraction. 2.5% isoflurane in 95% $O_2$-5% $CO_2$ at a rate of 1.0L/min was used to then anesthetize the mice. Next, mice were then laid in the supine position and anesthesia was decreased to and maintained at 2%. Anesthesia was monitored by repeated hind limb pinches in order to test for any response. Rapid thoracotomy was performed via midline sternotomy. Hearts were excised and rinsed of blood in PBS (50mM $NaH_2PO_4$, 150mM NaCl), then blotted dry and weighed on a tared dish to the nearest hundredth place. Whole hearts were flash frozen in liquid nitrogen and stored at -80°C until needed. These extracted tissues were then placed in a 2mL Dounce tissue grinder (Wheaton: 358132, 358133) in 1mL of cold homogenization buffer (25mM Tris·HCl, 150mM NaCl, 1mM EDTA; supplemented with 2:1,000 protease inhibitor cocktail). Tissues were ground until homogenous. Tissue lysates were centrifuged for 15 minutes at

14,000 RPM at 4°C. Finally, the supernatant (soluble protein layer) was extracted and stored at -80°C.

*Protein Assay*:

Pierce BCA protein assay kit (Thermo Fisher cat #: 23225) was be used to quantify proteins in the whole heart β2 Spectrin cKO lysates as well as the control whole heart lysates per kit protocol. Briefly, lysates were diluted to working concentration (for whole heart lysate, 1:20) and mixed 1:50 with Pierce BCA Protein Assay Reagent B to Pierce BCA Protein Assay Reagent A (Thermo Fisher: #23225). Following a 30-minute incubation at 37°C, the colormetric assay was read using endpoint spectrophotography on a spectrogram (Molecular Devices: SpectraMax M2) at 562nm. The 9 standards were twice replicated, and each sample thrice replicated.

*DNA extraction:*

DNA was extracted using Extracta DNA Prep (Quantabio: # 97065-350) per kit protocol. Briefly, the ear clippings were added to 50µL of extraction buffer, provided in the kit, and heated at 95°C for 30 minutes. Following this, 50µL of stabilization buffer were added to each sample that were cooled and placed in -20 °C for storage or further use.

*PCR and Gel Electrophoresis*:

Once extracted and stabilized, DNA was combined with AccuStart II PCR SuperMix (Quantabio: #76047-072) and forward and reverse primers per the following:

| Reagent: | Volume: |
|---|---|
| Forward Primer flox (GTAGCCTCCTTTCTGGGATGCCTGTGTATTT) | 0.75µL |

| | |
|---|---|
| Reverse Primer flox (CCCTTCCATGGTCTCATGTAAGCGGCAGAA) | 0.75µL |
| Accustart | 11.25µL |
| Cre primers:<br>Forward (ATGACAGACAGATCCCTCCTATCTCC)<br>Reverse (CTCATCACTCGTTGCATCATCGAC) | 0.7µL |
| DEPC | 3.25µL (flox), 4.3µL(Cre) |
| Extracted DNA | 2µL (flox), 1.75µL(Cre) |

Reaction tubes were place into the thermocycler (AppliedBiosystems: 2720). In the flox PCR protocol, the thermocycler will stay at 95 °C for 3 minutes, then begin 34 cycles of denaturation (95°C for 30 seconds), annellation (61°C for 30 seconds), and elongation (72°C for 30 seconds). Following this, the machine will hold at 72°C for 5 minutes then cool to 12 °C until the protocol is terminated. In the Cre protocol, the thermocycler will stay at 94°C for 2 minutes, then accomplish 10 cycles of denaturation (94°C for 20 seconds), annellation (65°C for 15 seconds), and elongation (68°C for 10 seconds). This will be followed by 28 cycles of denaturation (94°C for 15 seconds), annellation (60°C for 15 seconds), and elongation (72°C for 10 seconds). Next, the machine will hold at 72°C for 2 minutes, then cool to 10°C until the protocol is terminated. An agarose gel was made using 10µL ethidium bromide added to a 2g agarose/100mL TAE (Tris base, acetic acid and EDTA) solution. The samples were added into wells and electrophoresed at 100V for 30-45 minutes until clear separation of bands was apparent. Imaging of the bands was accomplished using ImageLab software (Bio-Rad: version 6.0.0, 2017, Hercules, CA, USA).

*Immunoblotting*:

4X Laemmli buffer (Bio-Rad: #161-0747) was added to specific tissue lysate concentrations that were determined by the protein assay and electrophoresed at 150V for 45-60 minutes in 4-15% precast TGX Stain-Free™ gels (Bio-Rad: #456-8084) using the Mini-PROTEAN tetra cell (Bio-Rad) until the band reached the bottom of the gel. After this, the electrophoresed gels were transferred to a nitrocellulose membrane (Bio-Rad: #162-0115) at 95V for 90 minutes using the Mini-PROTEAN tetra cell (Bio-Rad). Ponceau S stain (Cell Signaling: #59803) was used to confirm protein transfer where proper cuts were made around the nitrocellulose membrane. Membranes were destained with three washes with TBST (50mM Tris, 150mM NaCl, 0.1% Tween-20) for ten minutes. Next, membranes were blocked in 2.5% bovine serum albumin (BSA) for one hour at room temperature. Membranes were incubated with primary antibody overnight at 4°C. The next day, membranes were washed 3 times with TBST for 10 minutes. Membranes were incubated with secondary antibody for 2 hours at room temperature. ECL substrates (Thermo Fisher: PI134076, PI34096) were used for the chemi reaction with secondary antibodies. Densitometric analyses were performed using Image Lab software (Bio-Rad). For all experiments, protein values were normalized to a total protein loading control, GAPDH, or Vinculin.

*Antibodies*:

Antibodies used in immunoblotting and for immunofluorescent confocal microscopy were as follows: Smad2 (Cell Signaling: #Q15796), Smad3 (Abcam: ab40854), Smad2/3 (Invitrogen: #PA5-17621), Smad4 (Thermo Fisher: 4G1C6), Smad7 (Thermo Fisher: 42-0400), Collagen1

(Abcam: ab34710), TGFβ-1 (Abcam: ab92486), TGFβ-2(Abcam: ab36495), TGFβR1 (Sigma-Aldrich: #SAB4502958), TGFβR2 (Sigma-Aldrich: #AV44743), β2 Spectrin (Covance, custom homegrown), GATA4 (Abcam: ab84593), GAPDH (Invitrogen: #AM4300), and Vinculin (Invitrogen: #MA5-11690).

*Immunofluorescence:*

Immunofluorescent microscopy was done using isolated cardiomyocytes that were stored in 100% EtOH at -20 °C. Cells were suspended into the solution, aliquoted and spun down at 600RPM for 8 minutes. The supernatant was removed, and the cells were blocked in 500µL fish block (PBS, 3% cold water fish skin, 0.1% Triton X-100, 1% DMSO) for 1 hour at 4°C. The cells were spun down, blocking solution removed, and the target stain and counterstain of an opposing species were applied at 4°C overnight. Counterstains included α-actinin and desmin as controls for localization changes. Primary antibodies were removed in the supernatant the next day and 3 washes with fish block were performed. Following this, the secondary antibody was applied matching the target stain to 488 green and the counterstain to 568 red (AlexaFluor: Invitrogen #2026157, #1964370, #1981155, #1975519). These cells were incubated at 4°C for 2 hours wrapped in foil as to minimize light exposure. Next, the secondary antibody was removed and 3 washes in fish block were performed. Finally, the cells were mounted on slides in VECTASHIELD mounting media (Vector Labs: H-1200). Slides were stored preventing light exposure at 4°C until imaging via confocal microscopy.

*Confocal Microscopy:*

Images were acquired while at room temperature using a LSM 780 AxioObserver microscope (Zeiss) (40x 1.30 NA lens, pinhole = 1.0 airy disc). Images were then analyzed using ZEN imaging software (Zeiss). Researcher blinded to mice genotype acquired and analyzed images using identical detector gain and acquisition settings for all samples.

*Cell Culture*:

HL-1 (Sigma Aldrich: #SCC065) were plated and grown to confluency in 2mL/well of supplemented Claycomb media. Supplemented media was created using 435mL unsupplemented Claycomb media (Sigma Aldrich: #51800C) with 5mL penicillin (EMD Millipore: #TMS-AB2-C), 50mL HL-1 Qualified FBS (EMD Millipore: #TMS-016-B) and 5 mL 200mM L-Glutamine (EMD Millipore: #TMS-002-C). The cells were plated on a 1.5mL Gelatin/Fibronectin ECM matrix taken from a total solution of 10mL of 0.1% Gelatin Solution (EMD Millipore: #ES-006) in 40mL of Ultrapure water with 250uL Fibronectin (Sigma Aldrich: F-1141). Once confluent, the cells were lysed and stored in -80°C.

*TGF-B stimulation protocol:*

The HL-1 cells were stimulated for 72hours, changing the media every 24hours, with differing concentrations of TGFβ-1 recombinant protein (a kind gift from Dr. Accornero, OSU): untreated, 3ng/ml, and 10ng/ml. From this the cells were lysed and stored in -80°C until a protein assay was done followed by immunoblotting.

*Calpain Activity Assay:*

A calpain activity kit (Abcam: ab65308) was used to measure the amount of calpain cleavage per manufacturer instructions. Briefly, the HL-1 cells that had been stimulated with the TGF-B1 recombinant protein were harvested by washing the cells with cold PBS, suspending the cells in 100µL of Extraction Buffer and put-on ice for 20 minutes. The cells were mixed by gentle tapping during this incubation period. The samples were then centrifuged for 1 minute by microcentrifuge and the supernatant was transferred to a clean test tube. The assay was set up with duplicates for samples, standards and positive and negative controls. Additionally, Reaction Buffer and Calpain Substrate were added to each well per kit instructions. The assay was incubated at 37°C for 60 minutes. Finally, the reactions were analyzed (Fluorometric assay: measure Ex/Em = 400/505 nm) on a spectrogram (Molecular Devices: SpectraMax M2) and resulting figures were made.

Results:

***Validation of generated transgenic βII spectrin conditional knockout mice***

I set out to confirm that the LoxP sites flanking exon 3 of the β2 Spectrin gene (sptbn1), when crossed with mice expressing αMHC-Cre, would result in a cardiac specific loss of β2 spectrin. This was confirmed using PCR and gel electrophoresis and further validated in western blot analysis of brain, kidney, liver, and heart tissue lysates (not shown).

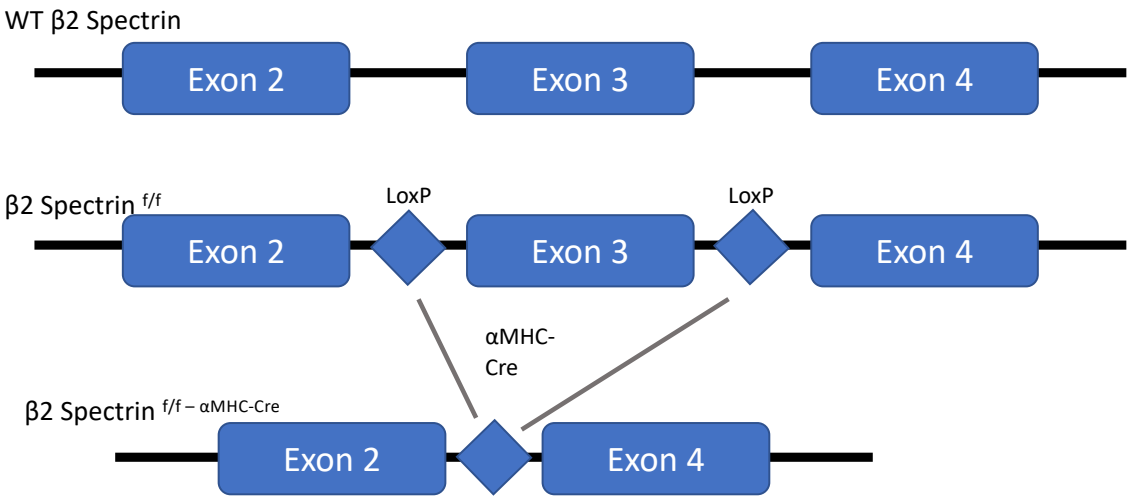
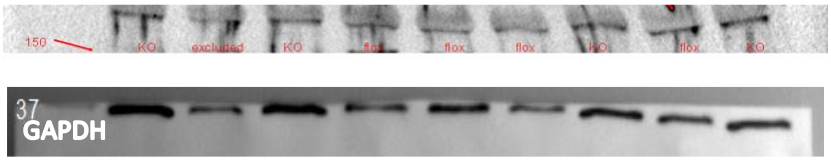



Figure 2: A model displaying the placement of the LoxP sites around exon 3 in order to disrupt the reading frame. When mice possessing the flox genes are crossed with mice possessing αMHC-Cre, the result is cardiac-specific knockout of β2 Spectrin.

***Impact of βII spectrin protein loss on downstream transcriptional product and cooperative TF***

I set out to determine the effects of the loss of β2 spectrin on a downstream product of SMAD-related transcription as well as the cooperative transcription factor, GATA4. I hypothesized that in the loss of β2 spectrin there will be a significant decrease in the fibrotic protein Collagen 1 and increase in GATA4.

The immunoblot analysis shows a significant decrease in the expression of Collagen 1 (Figure 3A). Further, it was seen that there is a significant increase in the downstream transcription factor GATA4 (Figure 3B), consistent with the hypothesis.



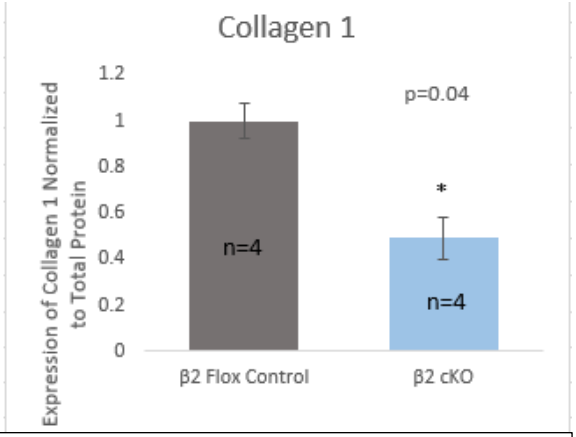
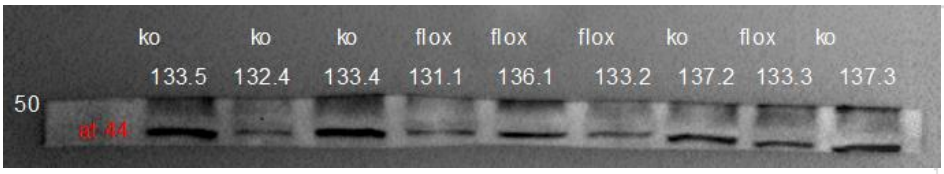
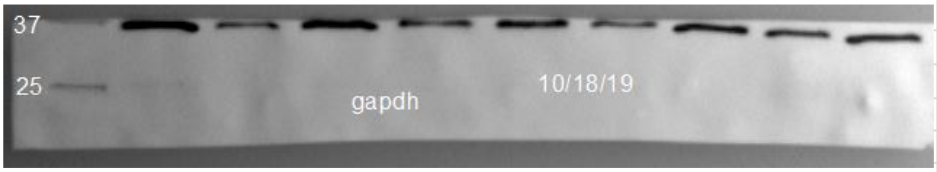




Figure 3A. Immunoblot analysis of Collagen 1 in the flox mice versus B2 spectrin cKO mice. In the analysis it was seen that there is a statistically significant decrease in the β2 spectrin cKO mice versus the flox control mice.



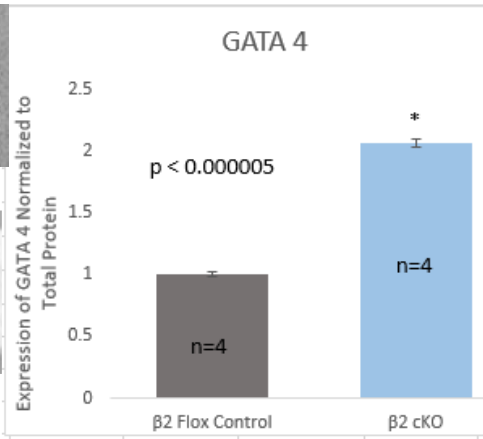
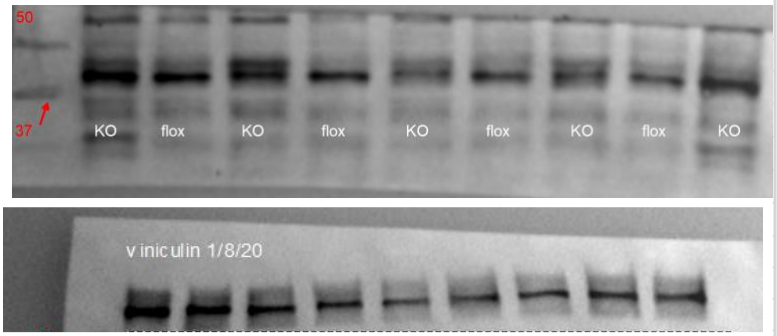



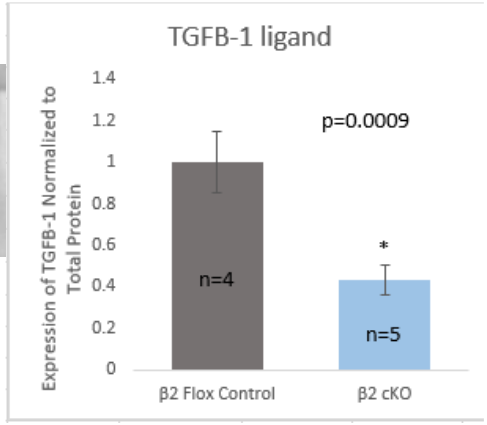

Figure 3B. Immunoblot analysis of GATA4, a downstream transcription factor known to work in cooperation with the TGFB signaling pathway. It was shown that there is a significant increase in the expression of GATA4.

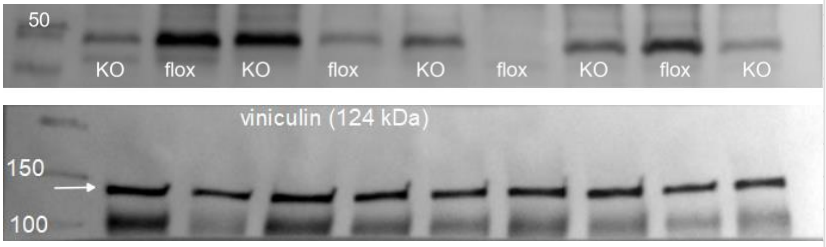
***Impact of βII spectrin protein loss on upstream cytokine and associated receptor activity***

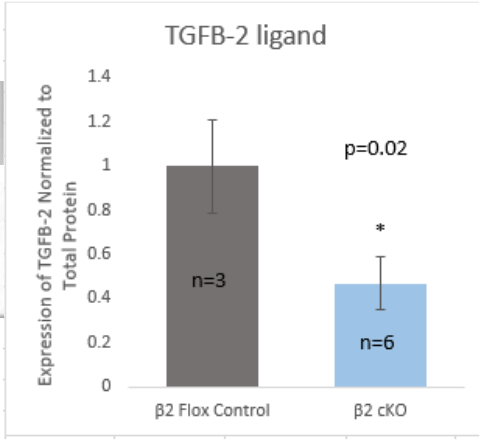
Through immunoblot analysis and immunofluorescence, I set out to determine if loss of β2 spectrin would result in differential expression of upstream cytokines (TGF-β1, TGF-β2) and their associated receptors (TGF-βR1, TGF-βR2). I hypothesized that there would be a significant decrease in expression of both the cytokine and the receptor in the whole heart lysates. It was seen that there is a significant decrease in TGF-β1 and TGF-β2 (Figure 4), consistent with the

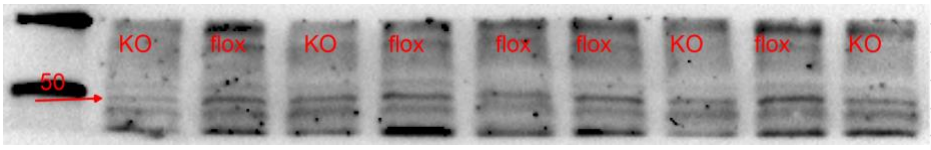
hypothesis. It was shown that TGF-βR1 trended toward a significant decrease, but it did not reach statistical significance. Contrary to the hypothesis, it was seen that TGF-β2 is significantly increased in the β2 spectrin cKO model.

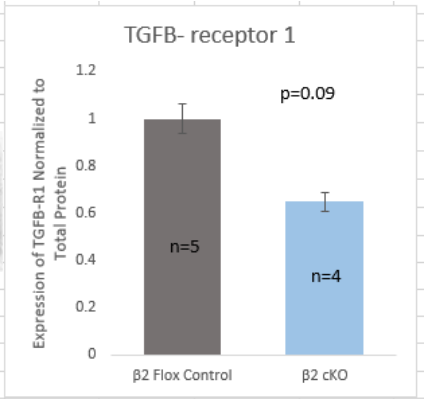


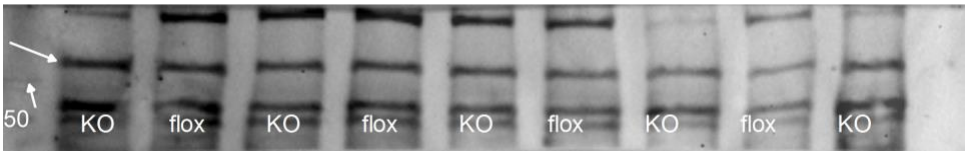





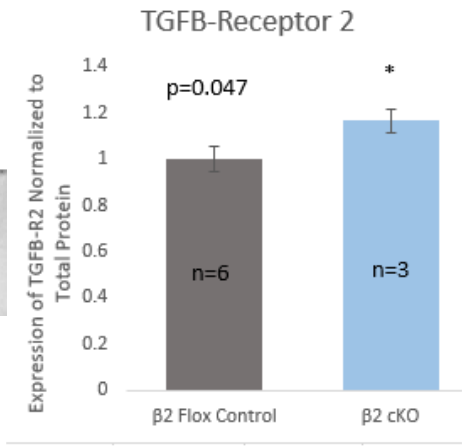




Figure 4. Triplicated western blots of the TGF-β1 and TGF-β2 cytokines. It was seen in the loss of β2 spectrin that there is significant decrease in both TGF-β1 and TGF-β2, but a significant increase in the expression of the TGF-βR2 and TGF-βR1 trended downward but did not reach significance.

It is important to recognize how the transcriptional factors in the cascade may be affected by a loss of β2 spectrin, so I set out to determine this. I hypothesized that there would be reduced expression of the interacting transcription factors since they would not be able to translocate to the nucleus without β2 spectrin. The immunoblot analysis showed that there is no significant difference between the levels of expression of SMAD2, SMAD3, and SMAD4 between the β2 spectrin cKO and the WT. This suggests that β2 spectrin loss does not impact the production nor degradation of the SMAD transcription factors, inconsistent with my hypothesis.

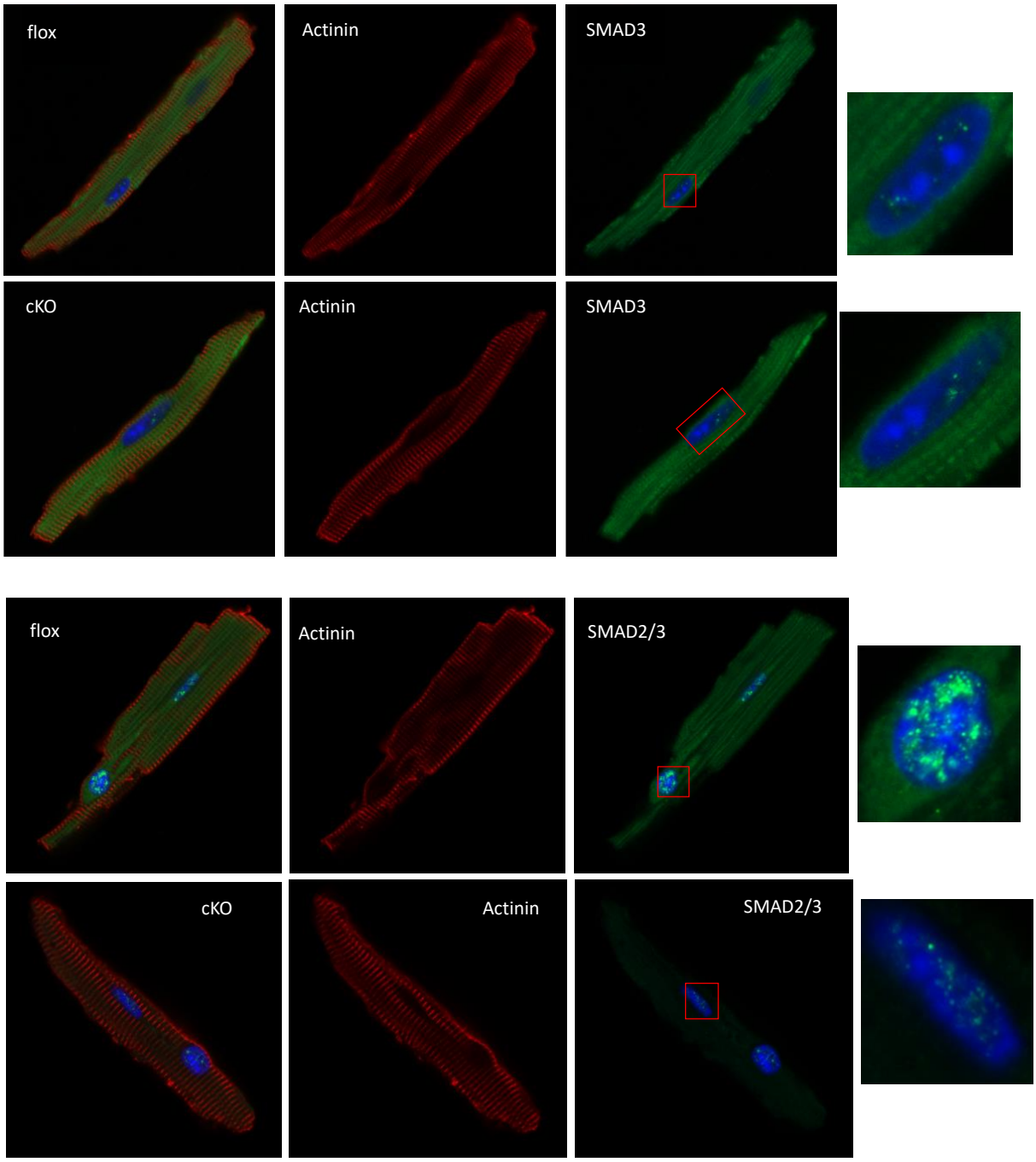



Figure 5. Immunofluorescence of SMAD3 and SMAD2/3 in isolated cardiomyocytes. There is no localization difference in SMAD3, but appearance of what seems to be less nuclear SMAD2/3 in the knockout model.

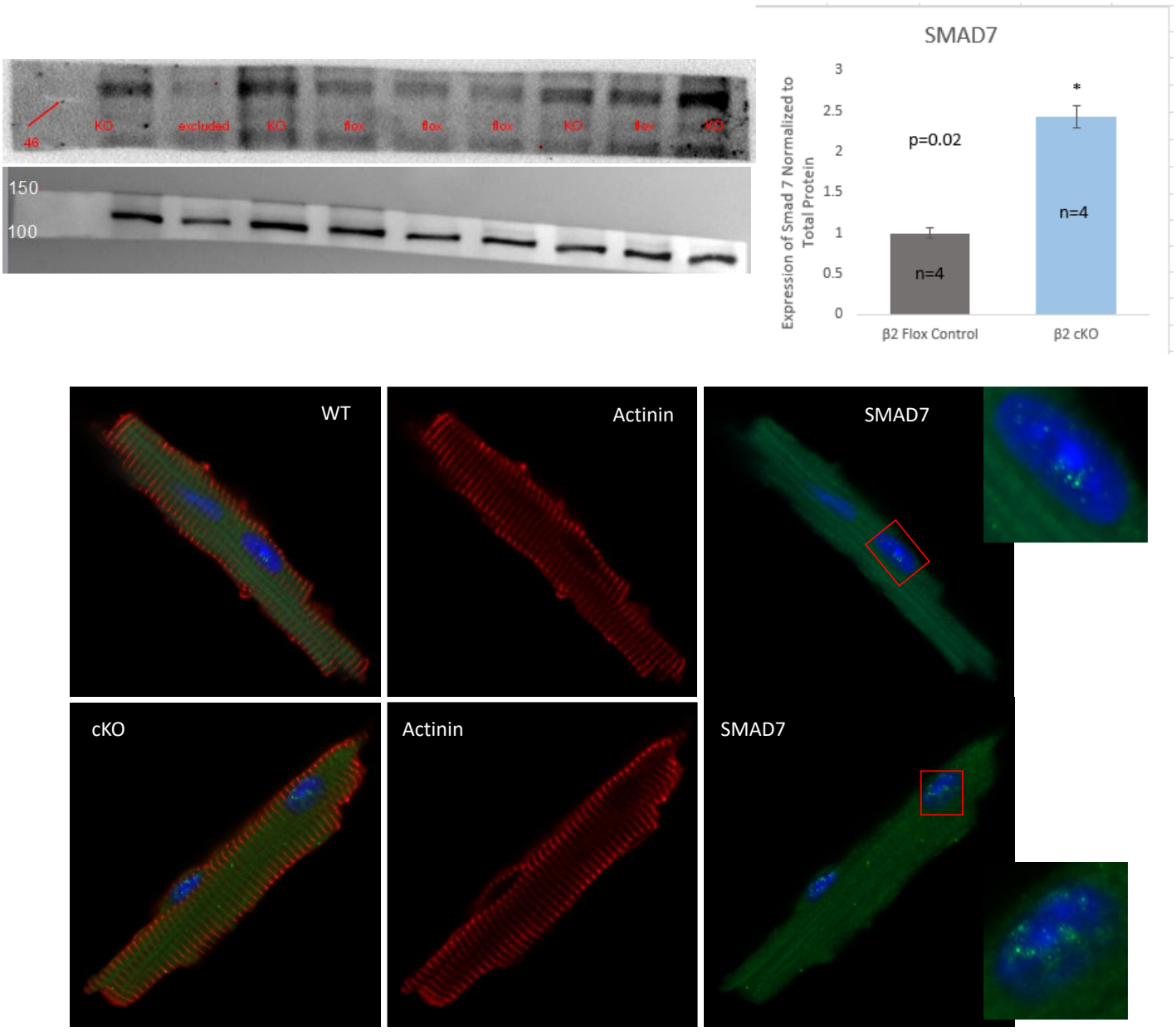



Figure 6. (Above) Western blot analysis indicates that there is a significant increase in expression of SMAD7 compared to the flox control. (Below) IF of SMAD7 with a zoom in on the nucleus indicates that perhaps more work needs to be done to quantify nuclear differences. Qualitatively it appears that there is a localization difference of SMAD7, the inhibitory SMAD.

It was seen in the western blot analysis that there is significantly increased expression of the inhibitory SMAD, SMAD7 (Figure 6). Additionally, in the IF, it appears that there is increased

nuclear localization of SMAD7. These results indicate that there is a decrease in TGF-β signaling as a result of the loss of β2 Spectrin, with the signaling pathway increasing inhibitory function.

***Impact of chronic TGF-β1 stimulation on calpain activity***

Critical to the hypothesis is that *chronic* TGF-β stimulation will result in increased calpain activity, and in turn that will result in increased β2 spectrin. This was tested in HL-1 cells using varying amounts of recombinant TGF-β1 protein: untreated, 3ng/ml and 10ng/ml. The calpain activity assay demonstrated that there is an increase in calpain activity with increasing concentrations of TGF-β1 protein (Figure 7). The HL-1 cell lysates in western blot analysis showed that there is a decrease in the full length β2 spectrin in the stimulated cells versus the untreated cells (Figure 9). While statistical significance was not reached, with an increase in the number of samples (n=3 versus n=5+) and replications, significance can be expected in future results. Additionally in the cell lysates, analysis of SMAD2/3 resulted in no significant difference between treated and untreated HL-1 cells.

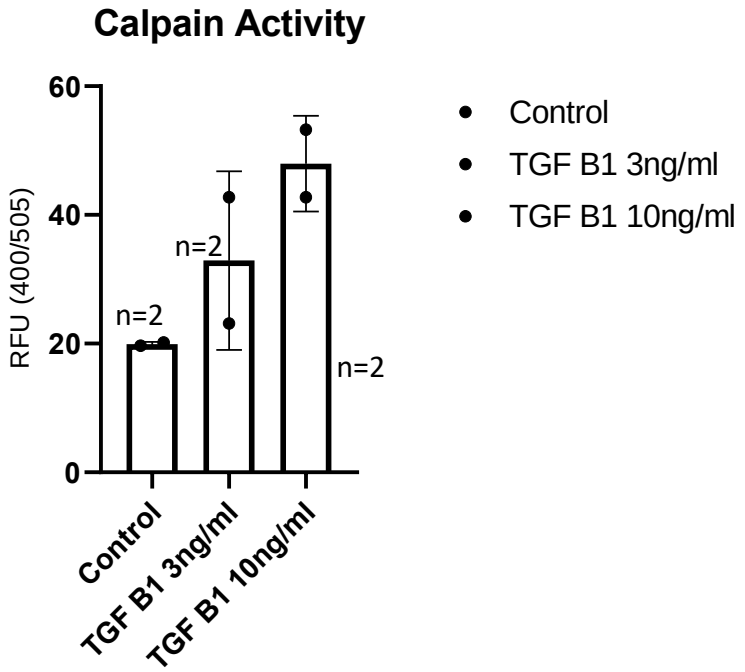



Figure 7. The calpain activity assay on the chronically stimulated HL-1 cells demonstrated that there is a dose-dependent increase in calpain activity depending on the concentration of TGFβ-1 protein. The lack of sample size leaves no significance in the results, but future studies will confirm that this trend is significant.

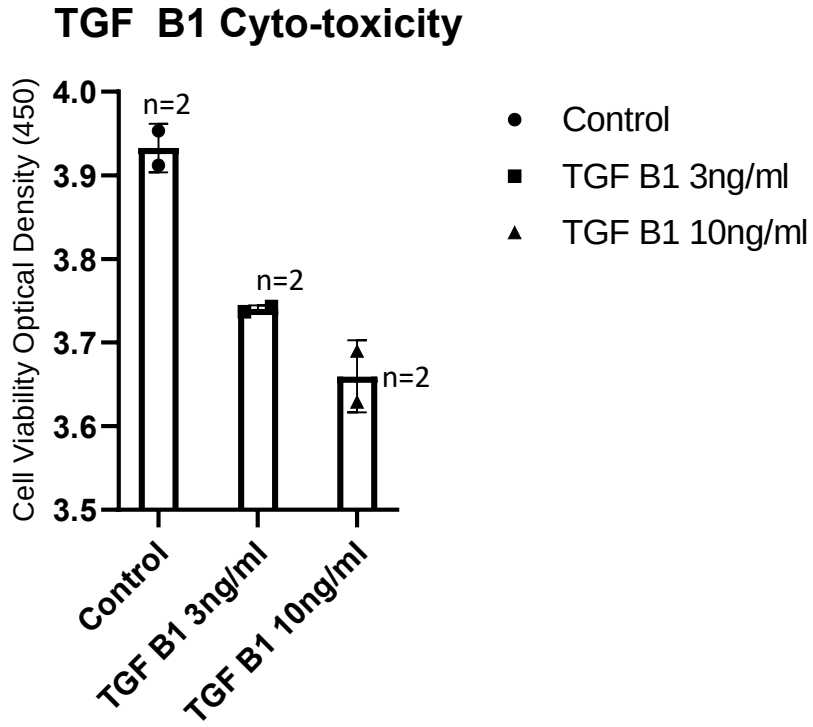
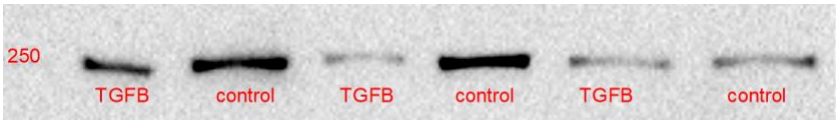



Figure 8. To show that the increase in calpain activity is not corresponding to cell death due to the TGF-β1 protein, a cyto-toxicity assay was performed. The results indicate that there is not a significant change between untreated HL-1 cells and 10ng/ml TGF-β1 protein, that of 7% change. These results indicate that there is little change in viability, and the calpain is a result of the chronic stimulation.



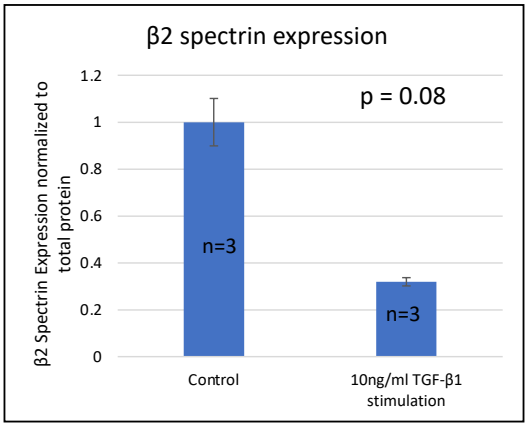




Figure 9. In the HL-1 cells that were treated with 10ng/ml of TGF-β1 protein, it was hypothesized that there will be increased β2 spectrin cleavage, represented by a decrease in the amount of full length β2 spectrin compared to the control. This is what was seen in the western blot analysis, consistent with the hypothesis.

Discussion:

The previous work of the Smith Lab has established β2 spectrin's regulatory role in cardiomyocyte membrane excitability and function. In that work, it was seen that the loss of β2 spectrin greatly affects the cytoskeleton of the cardiomyocyte and leads to accelerated HF.[12] It was building upon this and the work by Lim et al and their embryologic work investigating β2 spectrin as an adaptor protein in the TGF-β/SMAD pathway that this research thesis set out to accomplish.[20] To my knowledge, this is the first investigation into β2 spectrin in the TGF-β/SMAD pathway in the adult heart.

The necessity of β2 Spectrin in the TGF-β/SMAD pathway was tested through the transgenic cardiac-specific loss of β2 spectrin in an adult murine model. Importantly, it was seen that a critical downstream product of TGF-β signal transduction, Collagen 1, is significantly reduced in the knockout model. This protein makes up a majority of the extracellular matrix in a

fibrotic heart and is an excellent surveyor of this pathway.[21] While not a downstream product, GATA4 is a downstream transcription factor known to work cooperatively with the TGF-β/SMAD pathway. [22] Further, it has been demonstrated that GATA4 is antifibrotic in post-myocardial infarction mice, with emerging data that this, in addition to TGF-β suppression, could be a therapeutic strategy.[23,4] This provides downstream evidence that complete loss of β2 spectrin as an adaptor protein created an antifibrosis response.

While there was a significant decrease in the expression of TGF-β1 and TGF-β2 cytokines, there was no significant change in SMAD2, SMAD3, nor SMAD4 protein levels. These data are seemingly inconsistent. It could reasoned that there is no reason for SMAD degradation in the loss of β2 spectrin or perhaps that this simply not necessary in homeostasis in the adult mouse, but rather only for development. Additionally, it could be theorized that there would be a more significant molecular change in a stress model where the TGF-β signaling pathway is accentuated.

We now have greater understanding of the role of β2 spectrin in the TGF-β/SMAD pathway and a proposed mechanism by which β2 spectrin changes function from a cytoskeletal protein to an adaptor protein. In the loss of β2 spectrin, the canonical TGF-β pathway was dysregulated resulting in a decrease in both ligands and the key fibrotic transcriptional response, Collagen 1. Although there was no change in the expression of the associated SMAD proteins, the significant increase in inhibitory SMAD7 demonstrates that β2 spectrin is necessary for the canonical pathway. These data contradict the study by Lim et al that saw dysregulation in the SMAD proteins, although this was in complete loss of β2 spectrin prenatally. Further, the chronic stimulation of TGF-β1, as would be seen in a pathological state,

demonstrated that there is aberrant calpain activity, resulting in β2 spectrin cleavage in cardiomyocytes. These data provide a mechanism in heart failure that leads to an increasing fibrotic response and worsening heart failure.

These data are derived from a cKO model which present unique challenges when determining direct or indirect mechanistic effects from the loss of β2 spectrin. Enhanced by the complex interplay that the TGF-β pathway has with many other signaling pathways, there may be many potential mechanisms that could rescue or alter expression of the SMAD proteins. This can be seen in the non-canonical SMAD pathway with involvement with the BMP receptors, NODAL receptors, and ActvininR1 and ActvininR2. Hence, it is important to derive what changes occur at what time point due to the loss of β2 spectrin. We will be able to accomplish this through two different experimental methods, siRNA of β2 spectrin *in vitro* and a Mer-Cre-Mer mouse model with Tamoxifen. The first method has strength in the control over the cellular conditions and shorter time required to obtain meaningful data. The Mer-Cre-Mer mouse model finds strength in being more applicable to whole heart loss of β2 spectrin, understanding the physiological changes that occur, as well as translating potential findings to patients. The utility is lessened by the time and expense needed to generate the model. Thus, there is greater utility in a combination of these experimental methods.

There are a few limitations of this study design. First, there was no HF model used for the determination of differences in the nuclear translocation of β2 spectrin in disease state. Secondly, it could be considered a limitation that no cardiac fibroblasts were used for the *in vitro* TGF-β stimulation. This would be a better cell type to confirm the mechanism of pathological fibrosis in heart failure versus the atrial cells that were used. The data gathered in

the HL-1 cells remain important as any structural changes that result from TGF-β stimulation and calpain activation could lead to future arrhythmias and the activation of calpain would remain similar irrespective of cardiac cell type. These data could be used in tandem to further test the hypothesis and will be considered in the future directions. Third, there was no positive inhibitor used in the TGF-β stimulation to mechanistically prove that the recombinant TGF-β1 protein directly resulted in calpain activation through interaction with its receptor.

Future Direction:

There is much work to be done to fully understand the larger picture of this complex signaling pathway and its interactions with a cytoskeletal protein in pathology. The use of a TGF-β1 receptor inhibitor (Galunisertib) as a positive control in the calpain assay would fully ensure that it is the TGF-β1 causing the increased calpain activity and cleavage. Additionally, it would be helpful to add HF mouse model studies (TAC/SHAM) to study the effects of β2 spectrin knockout on this pathway and the potential for the other fibrotic pathways to be compensatory. Further, the HF mouse model would be leveraged to ascertain if a calpain inhibitor will prevent further fibrotic development. Masson's trichrome staining of the β2 spectrin cKO murine hearts is also a future direction for fibrotic assessment. The ultimate goal of this investigation is to turn to translational research, and this should be accomplished through human heart failure tissue samples to confirm the consistency between the model system and the goal.

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